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Author(s)	YAMADA, Jiro; TANBA, Hiroyuki; IZAWA, Masao
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PURIFICATION AND CHARACTERIZATION OF A DEBRANCHING ENZYME OF GERMINATED RICE SEEDS*

Jiro YAMADA, Hiroyuki TANBA**
and Masao IZAWA

Department of Agricultural Chemistry, Faculty of Agriculture,
Hokkaido University, Sapporo 060, Japan

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Introduction

Until now, several investigators have studied on debranching enzymes in germinated cereal seeds, and these enzymes have been reported to be involved in the degradation of starch during germination^{2,5,6}. In contrast with this, two of the present authors reported that high debranching activity was observed at the milk stage of rice seeds, where starch was exclusively synthesizing¹². Therefore, it must be meaningful that properties of the debranching enzymes in germinating and ripening rice seeds are compared with each other.

In previous studies of this series,^{12,13,14} a milky-stage enzyme could be purified and characterized. This paper deals with the purification and characterization of a debranching enzyme of germinated rice seeds.

Materials and Methods

Germination of rice seeds

Non-glutinous rice seeds (*Oryza sativa* L. c. v. "Yukara") were harvested at the experimental fields of the Hokkaido National Agricultural Experiment Station (Hitsujigaoka, Sapporo) in 1976. The rice seeds previously soaked over night in tap water were spread and submerged with water on large trays, and allowed to germinate at 26°C in the dark. Water was changed every day. At the 8th day of the germination, the seedlings were collected, washed with water and stored at -20°C.

* Studies on Rice Debranching Enzyme. Part IV. For Part III, see reference (14).

** Present address, The Research Laboratories of Kirin Brewery Co., Ltd., Takasaki 370-12, Japan.

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Reagents

Beta-amylase (Wako Pure Chemicals Industry, Ltd.), pancreatic α -amylase and phosphorylase *a* (Boehringer-Mannheim, GmbH) were obtained from commercial sources. To purify the rice enzyme, DEAE-Sephadex A-50 (3.5 ± 0.5 meq/g) and Sephadex G-200 (particle size $40 \sim 120 \mu$) (Pharmacia Fine Chemicals AB) were used. For determination of the molecular weight of the enzyme, reference proteins purchased from Boehringer-Mannheim, GmbH, were used.

Substrates

Glutinous and non-glutinous rice starches were prepared from matured rice seeds of c. v. "Kamui-mochi" and of "Horyu", respectively. Potato amylose and non-glutinous rice amylopectin were obtained by a modified Schoch's method¹¹ from commercial potato starch and non-glutinous rice starch. Phytoglycogen was prepared from sweet corn at the milky stage (c. v. "Golden Cross Bantam") by extraction with water followed by deproteinization with chloroform. Various β -limit dextrans were obtained by repeated β -amylolyses of glutinous rice starch, non-glutinous rice amylopectin and sweet corn phytoglycogen. Phosphorylase limit dextrans were prepared from glutinous starch and phytoglycogen by phosphorylase *a*. Commercial soluble starch (Merck and Co., Inc.) and soluble starch prepared from glutinous rice by Lintner's method¹⁰ were also used. A limit-hydrolyzate obtained from the commercial soluble starch by pancreatic α -amylase was subjected to column chromatography [charcoal-Celite (1 : 1)], and a fraction eluted with 20% ethanol (α -amylolysis product) was employed as one of the substrates. Isomaltose and panose were the gifts by courtesy of Dr. S. CHIBA of this university. Pullulan (mol. wt. 62,000) was purchased from Hayashibara Co., Ltd.

Assay for proteins and reducing sugars

Protein was determined by the method of LOWRY *et al.*⁴ and calibrated for milk casein. Proteins eluting from the columns were monitored by 280-nm absorption. Reducing sugars were determined by the Somogyi-Nelson method^{8,9}. Concentration of the substrates was calculated from the reducing power obtained after hydrolysis with 1 N sulfuric acid in boiling water for 2.5 hr.

Assay for enzyme activity

Activity of the debranching enzyme was measured in an incubation mixture (1 ml) of enzyme (0.2 ml), pullulan (0.2%, 0.5 ml) and McIlvaine buffer

(pH 5.6, 0.3 ml). After the incubation at 25°C for 10 min, the reaction was stopped by addition of Somogyi's copper reagent (1 ml) and reducing power was determined. The debranching activity is expressed in terms of pullulanase activity, whose 1 unit is defined as the amount of pullulanase which increases the reducing power equivalent to 1 μ mol of maltotriose per min.¹²⁾

Saccharifying activity was measured by the use of 1% soluble starch (0.5 ml) instead of pullulan. Its 1 unit is defined as the amount of the enzyme which increases the reducing power equivalent to 1 μ mol of maltose per min.

For assaying of liquefying activity, a mixture of enzyme (0.2 ml), amylose (0.2%, 0.5 ml) and McIlvaine buffer (pH 5.6, 0.3 ml) was incubated at 25°C for 10 min. After the reaction was terminated with 6% acetic acid (1 ml), a solution of 0.2% iodine in 2% potassium iodide (1 ml) was added to the mixture and then diluted to 50 ml with water. A portion of this solution was assayed for 660-nm extinction of the iodine-amylose complex. One unit of the liquefying activity is defined as the enzyme amount which decreases the extinction by 1% per min under the conditions described above.

Kinetic studies were conducted at 30°C.

Thin-layer chromatography

A solvent system used for thin-layer chromatography [Silica gel G, Type 60 (Merck), 0.25 mm thick] is a mixture of ethyl acetate-methanol-water (52:36:13, v/v)⁹⁾. As a spray reagent for sugar detection, 50% sulfuric acid was employed.

Disc electrophoresis

Enzyme protein was disc-electrophoretically run on a 7.5% polyacrylamide gel for 60 min at pH 9.4 and 2 mA per tube⁷⁾. Location of the proteins was visualized with Amido Black 10 B. For enzyme activity assay, each of the 2-mm sections cut off from the gel was put into McIlvaine buffer (pH 5.6, 2 ml) and thoroughly dissolved by standing over night at 0-4°C. A mixture of 0.2% pullulan (0.5 ml) and one of the solutions thus prepared (0.5 ml) was incubated at 25°C for 60 min, and then reducing power was measured by the Somogyi-Nelson method.

Results

Purification of enzyme

Germinated rice seeds [seedlings (5.4 kg) germinated from unhulled rice seeds (2 kg)] were homogenized with 0.1 M sodium phosphate buffer (pH 7.2, 10.8 liter) in a mixer. The homogenate was centrifuged at 10,000 \times g

for 15 min. Proteins in the supernatant ("Crude extract") were precipitated by the addition of solid ammonium sulfate to 70% saturation. The precipitates resulted were dissolved in 0.1 M sodium phosphate buffer (pH 7.2) and centrifuged at $10,000 \times g$ for 30 min. The supernatant was diluted to 1 liter with the same buffer ("Crude enzyme"). To this solution, a solution fully saturated with ammonium sulfate was added stepwise. Most of the debranching activity could be recovered in the protein fraction precipitating between 0.3 and 0.4 saturation. The precipitates obtained from this fraction were dissolved in sodium phosphate buffer (pH 7.2, 100 ml) ("ammonium sulfate ppt.").

"Ammonium sulfate ppt." was repeatedly dialyzed against 0.01 M sodium phosphate buffer containing 0.1 M sodium chloride (pH 7.2) and the resulting precipitate was discarded by centrifugation. The supernatant ("Dialyzed solution") was applied to a column of DEAE-Sephadex A-50 (3.2×32 cm)

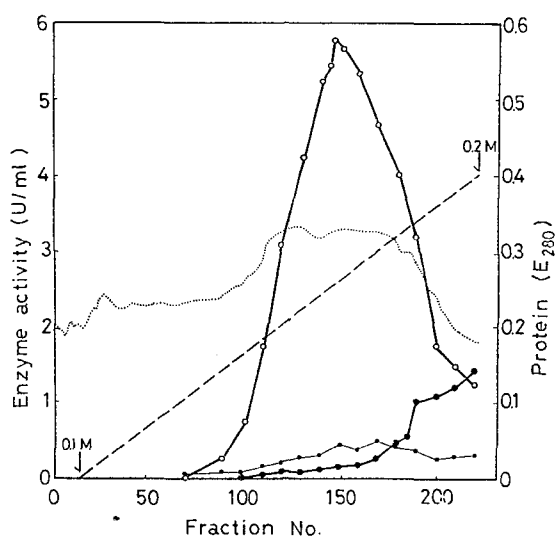


Fig. 1. DEAE-Sephadex A-50 Column Chromatography of Debranching Enzyme from Germinated Rice Seeds.

Protein (2.51 g, pullulanase activity 2970 U) was applied to a column (3.2×32 cm) of DEAE-Sephadex A-50 equilibrated with 0.01 M sodium phosphate buffer containing 0.1 M sodium chloride (pH 7.2). Gradient with sodium chloride from 0.1 to 0.2 M was used to elute the enzyme. Twenty-ml fractions were collected at a flow rate of 20 ml per hr.

—○—: Pullulanase activity ($\times 10^{-1}$), —●—: liquefying activity ($\times 10^3$), —●—: saccharifying activity ($\times 10^{-1}$),: protein, and ----: sodium chloride concentration.

equilibrated with the same buffer. The column was washed with the same buffer to remove unadsorbable proteins and then eluted by a linear gradient of sodium chloride from 0.1 to 0.2 M (Fig. 1). Fractions No. 100-177 showing

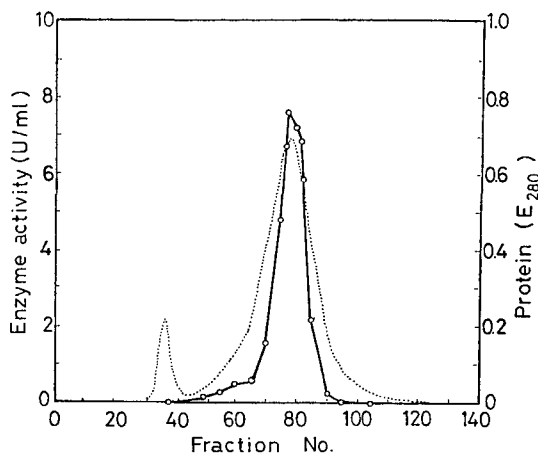


Fig. 2. Sephadex G-200 Gel Filtration of Debranching Enzyme from Germinated Rice Seeds.

Protein (46.8 mg, pullulanase activity 397 U) was applied to a column (2.6 × 95.5 cm) of Sephadex G-200. Elution was conducted with 0.05 M sodium phosphate buffer containing 0.25 M sodium chloride and 4-ml fractions were collected.

—○—: Pullulanase activity,: protein.

TABLE 1. Purification process of debranching enzyme from germinated rice seeds*

Preparation	Volume (ml)	Protein (mg)	Pullulanase			Liquefying activity		Saccharifying activity	
			Total activity (U)	Recovery (%)	Specific activity (U/mg)	Activity (U)	Recovery (%)	Activity (U)	Recovery (%)
Crude extract	11,450	32,600	4100	100.0	0.126	8,770,000	100.0	7250	100.0
Crude enzyme	2,580	12,800	4330	105.6	0.338	4,560,000	52.0	7100	97.9
Ammonium sulfate ppt.	110	3,020	2760	67.3	0.914	1,400,000	16.0	3060	42.2
Dialyzed solution	160	2,510	2970	72.4	1.18	1,600,000	18.2	2840	39.2
DEAE-Sephadex A-50 enzyme	3.3	135	666	16.2	4.93	1,800	0.0205	31.3	0.432
Sephadex G-200 enzyme	2.7	46.8	397	9.7	8.48	468	0.0053	18.0	0.248
Purified enzyme	2.0	23.0	230	5.6	10.00	119	0.0014	6.7	0.092

* Germinated rice seeds (c. v. "Yukara", 5.4 kg) were used.

high pullulanase and low liquefying activities were pooled and concentrated with an Amicon ultrafiltration cell equipped with a Diaflo PM-30 membrane and then with a collodion bag ("DEAE-Sephadex A-50 enzyme").

This concentrate was further purified by gel filtration on a Sephadex G-200 column (2.6 × 95.5 cm) with 0.05 M sodium phosphate buffer containing 0.25 M sodium chloride (pH 7.2). The eluates showing debranching activity were combined and concentrated in a similar manner as above ("Sephadex G-200 enzyme"). This concentrate was re-chromatographed on another Sephadex G-200 column (Fig. 2). Fractions No. 71-92 were collected and concentrated to give the final enzyme preparation ("Purified enzyme").

Table 1 shows the purification process.

Molecular weight

Molecular weight of the purified debranching enzyme was estimated to be ca. 70,000 from comparison with those of the reference proteins by Sephadex G-200 gel filtration (Fig. 3). This value was almost identical with that of the milky-stage enzyme (mol. wt., 69,000)¹⁵⁾.

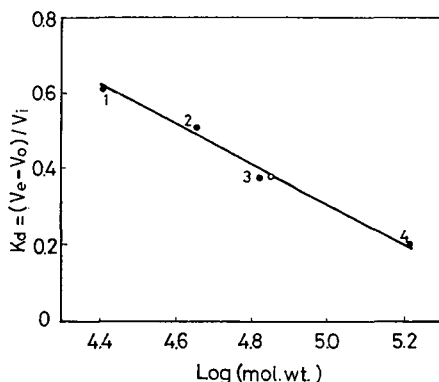


Fig. 3. Estimation of Molecular Weight of Debranching Enzyme from Germinated Rice Seeds by Sephadex G-200 Gel Filtration.

V_e , elution volume; V_o , void volume; V_i , inner volume.

Reference proteins (●): (1) chymotrypsinogen A (mol. wt., 25,000), (2) ovalbumin (45,000), (3) bovine serum albumin (67,000), and (4) γ -globulin (160,000). Rice debranching enzyme: (○).

Column size and eluting conditions: see Fig. 2.

Disc-electrophoresis

Disc-electrophoresis of the purified enzyme preparation was carried out on a polyacrylamide gel (pH 9.4). It was found that this enzyme preparation

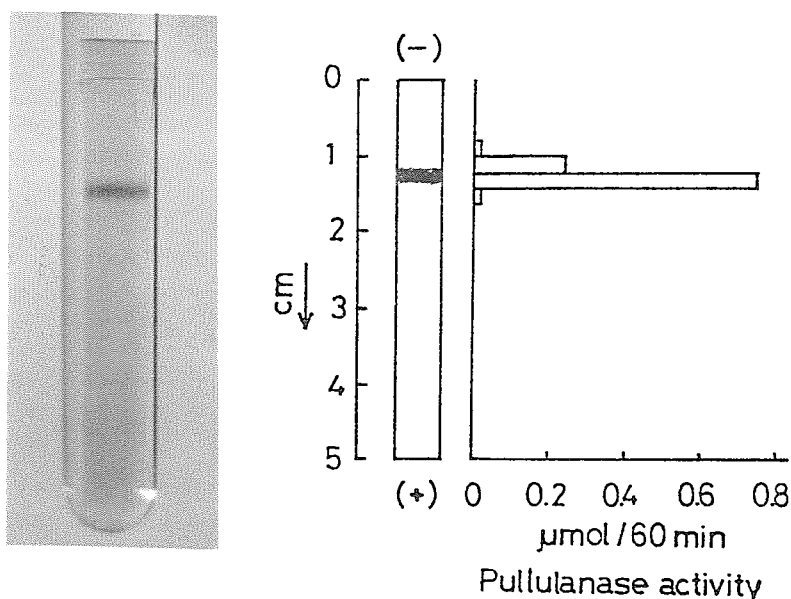


Fig. 4. Disc-electrophoresis of Debranching Enzyme from Germinated Rice Seeds.

Electrophoresis was performed on a 7.5% acrylamide gel at pH 9.4 and 2 mA per tube for 60 min. Proteins were located by staining with Amido Black 10 B.

For enzyme assay, each of the 2-mm sections cut off from the gel after electrophoresis was put into McIlvaine buffer (pH 5.6, 2 ml) and thoroughly dissolved by standing overnight at 4°C. A reaction mixture of the enzyme solution thus prepared (0.5 ml) and 0.2% pullulan (0.5 ml) was incubated at 25°C for 60 min and reducing power was measured by the Somogyi-Nelson method.

gave only a single protein band and the highest debranching activity was detected on this band (Fig. 4).

General properties

Optimum pH of the purified debranching enzyme was found to be 5.6 (McIlvaine buffer) (Fig. 5). These properties was almost the same as those of the milky-stage enzyme¹²⁾.

The enzyme was stable between pH 5 and 8 at 30°C and between pH 7 and 8 at 40°C (Fig. 6). Thermal stability of the enzyme was examined at pH 5.6. As shown in Fig. 7, it was perfectly stable at 25°C and stable at 40°C, whereas labile at 45–50°C and inactivated spontaneously at 60°C. Thus, the present enzyme was found to be somewhat more stable than the milky-stage enzyme¹⁵⁾.

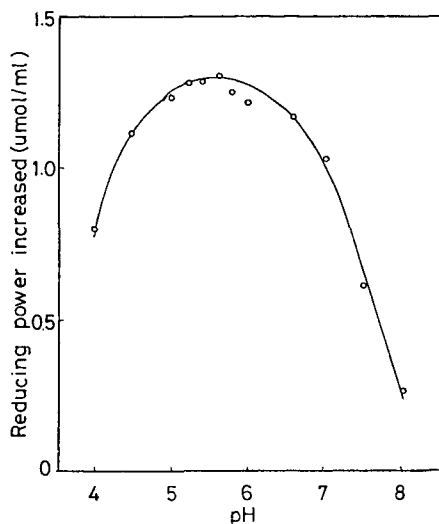


Fig. 5. Optimum pH of Debranching Enzyme from Germinated Rice Seeds.

A mixture of enzyme (0.2 ml, 0.087 U/ml), pullulan (0.2%, 0.5 ml) and McIlvaine buffer (pH 4.0~8.0, 0.3 ml) was incubated at 25°C for 15 min and reducing power was measured.

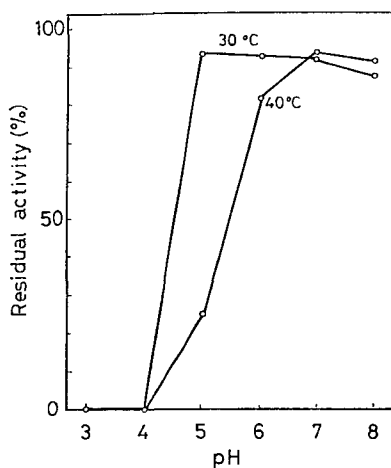


Fig. 6. pH-Stability of Debranching Enzyme from Germinated Rice Seeds.

A mixture of enzyme (0.2 ml, 0.63 U) and McIlvaine buffer (pH 3.0~8.0, 0.2 ml) was allowed to stand at 30°C or 40°C for 1 hr. Then, cold McIlvaine buffer (pH 5.6, 4.6 ml) was added to the mixture and its 0.5 ml portion was assayed for debranching activity to pullulan.

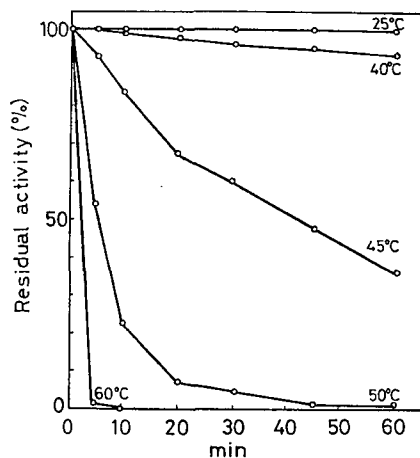


Fig. 7. Thermal Stability of Debranching Enzyme from Germinated Rice Seeds.

Immediately after the enzyme (0.073 U) in McIlvaine buffer (pH 5.6, 0.2 ml) was heated for scheduled periods at various temperatures, the solution was cooled and residual debranching activity to pullulan was determined.

Substrate specificity

Substrate specificity of the purified enzyme on several oligo- and polysaccharides was investigated. A reaction mixture (1 ml) of enzyme (0.0378 U) and one of the substrates (1 mg, except for panose and isomaltose 0.5 mg) was incubated at pH 5.6 and 25°C. The results are shown in Table 2.

TABLE 2. Action of debranching enzyme from germinated rice seeds on various substrates

Substrate	Reducing power (μ mol D-glucose/ml) increased after incubation for		
	10 min	30 min	60 min
Amylose (potato)	0	0	0
Amylopectin (rice)	0	0.020	0.036
Glutinous rice starch	0.012	0.035	0.069
Phytoglycogen (corn)	0	0	0
Phytoglycogen β -dextrin	0.012	0.033	0.056
Phytoglycogen ϕ -dextrin	0	0	0.011
Amylopectin β -dextrin	0.171	0.303	0.391
Glutinous rice starch β -dextrin	0.209	0.363	0.440
Pullulan	0.997	1.88	1.89
α -Amylolysis product	0.405	0.445	0.475
Panose	0.006	0.006	0.006
Isomaltose	0	0	0

A mixture (1 ml) of enzyme (0.0378 U) and substrate (1 mg/ml, except for panose and isomaltose 0.5 mg/ml) was incubated at pH 5.6 and 25°C.

Pullulan was most rapidly hydrolyzed and an α -amylolysis product was quickly debranched. Glutinous rice starch β -limit dextrin and amylopectin β -limit dextrin were also debranched, but glutinous rice starch, phytoglycogen β -limit dextrin and amylopectin were hydrolyzed very slowly. Amylose, phytoglycogen and its phosphorylase limit dextrin, as well as panose and isomaltose, were hardly or not hydrolyzed.

These substrate specificities were identical with those of the milky-stage enzyme¹²⁾.

Kinetics

Kinetics of the purified enzyme was studied at pH 5.6 and 30°C. Line-weaver-Burk plots are shown in Fig. 8. Michaelis constant (K_m) and relative maximum velocity (V_{rel}) read from Fig. 8 are also shown in Table 3. K_m -

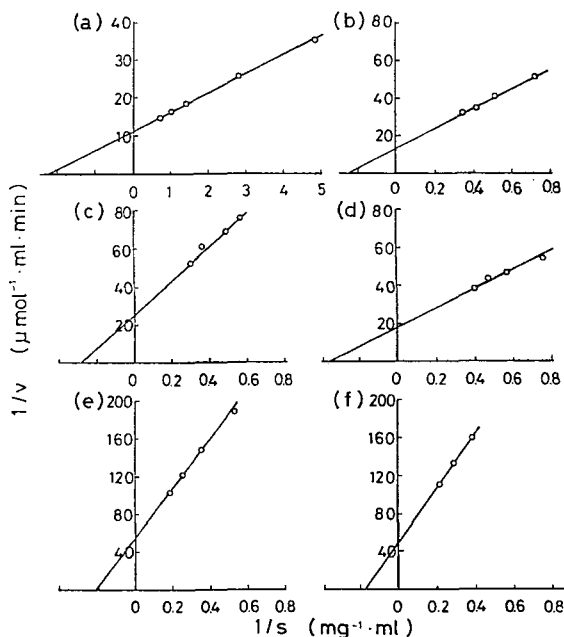


Fig. 8. Lineweaver-Burk Plots of Hydrolyses by Debranching Enzyme from Germinated Rice Seeds.

(a) Pullulan, (b) glutinous rice starch β -limit dextrin, (c) glutinous rice starch phosphorylase limit dextrin, (d) amylopectin β -limit dextrin, (e) glutinous rice soluble starch, and (f) soluble starch (Merck).

A reaction mixture of enzyme (0.05 U, 0.2 ml), one of the substrates (0.5 ml, various concentrations), and McIlvaine buffer (pH 5.6, 0.3 ml) was incubated at 30°C. Then, the digest was assayed for reducing power.

TABLE 3. Kinetic data for hydrolyses of various substrates by debranching enzyme from germinated rice seeds

Substrate	Enzyme from germinated seeds		Enzyme from seeds at milky stage ¹⁴⁾	
	K_m (mg/ml)	V_{rel} (%)	K_m (mg/ml)	V_{rel} (%)
Pullulan	0.44	119.0	0.22	127.0
Glutinous rice starch β -dextrin	4.08	100.0	3.33	100.0
Glutinous rice starch ϕ -dextrin	3.52	53.9	2.31	53.3
Amylopectin β -dextrin	2.78	73.3	1.57	81.9
Glutinous rice soluble starch	4.76	24.2	5.00*	10.5*
Soluble starch (Merck)	5.88	26.2	5.00**	7.0**

Incubation: see Fig. 8.

* Glutinous rice starch.

** Non-glutinous rice amylopectin.

values of the present enzyme were found to be somewhat larger than or similar to those of the milky-stage enzyme. V_{rel} -values were also almost identical between the two enzymes.

Mode of pullulan hydrolysis

Debranching products from the most effective substrate, pullulan, were investigated by silica gel thin-layer chromatography. As shown in Fig. 9, it was found that hexaose (6^3 -maltotriosylmaltotriose) and higher oligosaccharides appeared at an early stage of the hydrolysis and they gradually degraded to produce maltotriose. This suggests that the enzyme hydrolyzes pullulan on an endo-fashion. The milky-stage enzyme has been reported already to catalyze the hydrolysis of pullulan in such a way¹³.

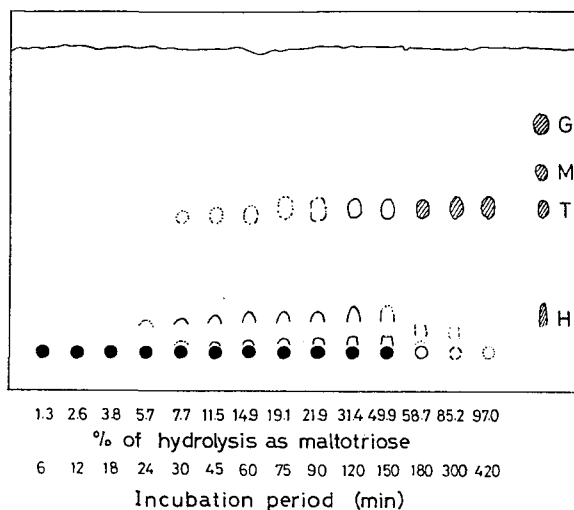


Fig. 9. Thin-layer Chromatogram of Hydrolyzates from Pullulan by Debranching Enzyme from Germinated Rice Seeds.

A mixture (1 ml) of enzyme (0.005 U) and pullulan (1 mg) was incubated at pH 5.6 and 30°C. After the hydrolyzate was dried *in vacuo* and dissolved in water (20 μ l), a portion of the solution thus prepared (10 μ l) was put onto a thin-layer plate and chromatographed with a solvent mixture, ethyl acetate-methanol-water (52:36:13, v/v). The chromatogram was sprayed with sulfuric acid (50%).

Reference sugars: G, D-glucose; M, maltose; T, maltotriose; H, 6^3 -0- α -maltotriosylmaltotriose.

Discussion

It is well known that germination induces the activity of hydrolyzing enzymes, especially of α -amylase, in cereal seeds. The α -amylase is able to hydrolyze α -1,4-linked D-glucose units in the molecules of starch but unable

to degrade α -1,6-linkages. Therefore, another type of enzymes, a so-called debranching enzyme, is required for the perfect degradation of starch. Debranching enzymes in the germinated seeds of barley^{5,6}, wheat⁶, rye⁶, sorghum²⁰ and maize⁶ and also those in the rice seeds at dormant¹¹ and milky stages¹² have been studied already. However, investigations on the rice seeds at germination stage have not been reported.

In the present study, a debranching enzyme could be purified 80 fold from the germinated rice seeds. High amylase activity existing in the seeds was able to remove effectively by DEAE-Sephadex A-50 and Sephadex G-200 column chromatography (Fig. 1 and Table 1). That is, liquefying, saccharifying and pullulanase activities recovered in the purified enzyme were 0.0014, 0.092 and 5.61%, respectively, and negligible amounts of the former two did not interfere with the determination of pullulanase activity.

When properties of this disc-electrophoretically homogeneous enzyme were compared with those of the milky-stage enzyme previously obtained,^{12, 13, 14, 15} these two enzymes were found to be almost identical. Consequently, whether the debranching enzyme was involved peculiarly or not in the synthesis of starch at the ripening stage could not be clarified.

Summary

A debranching enzyme was purified 80-fold from the germinated rice seeds by fractional precipitation with ammonium sulfate and DEAE-Sephadex A-50 and Sephadex G-200 column chromatographies. This disc-electrophoretically homogeneous enzyme (mol. wt., 70,000) showed the following properties: opt pH 5.6; stable at pH 5-8, 30°C and pH 7-8, 40°C; stable at 25-40°C, labile at 45-50°C, and inactivated at 60°C. By this enzyme, pullulan was most rapidly hydrolyzed in an endo-fashion and an α -amylolysis product from soluble starch was quickly debranched. Glutinous rice starch β -limit dextrin and amylopectin β -limit dextrin were also debranched, but glutinous rice starch, phytyglycogen β -limit dextrin and amylopectin were hydrolyzed very slowly. Amylose, phytyglycogen and its phosphorylase limit dextrin were hardly or not hydrolyzed. These properties were almost identical with those of the milky-stage debranching enzyme.

Literature Cited

1. DUNN, G. and MANNERS, D. J.: The limit dextrinases from ungerminated oats (*Avena sativa* L.) and ungerminated rice (*Oryza sativa* L.), *Carbohydr. Res.*, **39**: 283-293. 1975
2. HARDIE, D. G., MANNERS, D. J. and YELLOWLEES, D.: The limit dextrinase

- from malted sorghum (*Sorghum vulgare*), *Carbohydr. Res.*, **50**: 75-85. 1976
3. HUBER, C. N., SCOBELL, H. and TAI, H.: Determination of saccharide distribution of corn syrup by direct densitometry of thin-layer chromatograms, *Cereal Chem.*, **43**: 342-346. 1966
 4. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J.: Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, **193**: 265-275. 1951
 5. MACWILLIAM, I. C. and HARRIS, G.: The separation of limit dextrinase from R-enzyme and aspects of the activities of the separated enzymes, *Arch. Biochem. Biophys.*, **84**: 442-454. 1959
 6. MANNERS, D. J. and YELLOWLEES, D.: Studies on debranching enzymes. Part I. The limit dextrinase activity of extracts of certain higher plants and commercial malts, *J. Inst. Brew.*, **79**: 377-385. 1973
 7. NAGAI, Y.: Method of disc-electrophoresis (in Japanese), *Tanpakushitsu Kakusan Koso, Bessatsu, Seibutsukagaku Zikkenho IX*, p. 3-14. Kyoritsushuppan, Tokyo, 1967
 8. NELSON, N.: A photometric adaptation of the Somogyi method for the determination of glucose, *J. Biol. Chem.*, **153**: 375-380. 1944
 9. SOMOGYI, M.: Notes on sugar determination, *J. Biol. Chem.*, **195**: 19-23. 1952
 10. TAKAOKA, K.: Preparation of soluble starch (in Japanese), *Denpunkagaku, Zohoban*, ed. by NIKUNI, Z., p. 327-331. Asakura-shoten, Tokyo, 1955
 11. WILSON, Jr., E. J., SCHOCH, T. J. and HUDSON, C. S.: The action of *macerans* amylase on the fractions from starch, *J. Am. Chem. Soc.*, **65**: 1380-1383. 1943
 12. YAMADA, J. and IZAWA, M.: A debranching enzyme of rice seeds at milky stage, Its purification and substrate specificities, *Agric. Biol. Chem.*, **43**: 37-44. 1979
 13. YAMADA, J. and IZAWA, M.: Mode of action of a rice debranching enzyme on pullulan and its related oligosaccharides, *Agric. Biol. Chem.*, **43**: 2515-2521. 1979
 14. YAMADA, J. and KOJIMA, E.: Action mode of rice debranching enzyme on starch-like polysaccharides, submitted to *Agric. Biol. Chem.*
 15. YAMADA, J.: Unpublished data.